

The effects of aging on gene expression in the hypothalamus and cortex of mice

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A better understanding of the molecular effects of aging in the brain may help to reveal important aspects of organismal aging, as well as processes that lead to age-related brain dysfunction. In this study, we have examined differences in gene expression in the hypothalamus and cortex of young and aged mice by using high-density oligonucleotide arrays. A number of key genes involved in neuronal structure and signaling are differentially expressed in both the aged hypothalamus and cortex, including synaptotagmin I, cAMP-dependent protein kinase C β , apolipoprotein E, protein phosphatase 2A, and prostaglandin D. Misregulation of these proteins may contribute to age-related memory deficits and neurodegenerative diseases. In addition, many proteases that play essential roles in regulating neuropeptide metabolism, amyloid precursor protein processing, and neuronal apoptosis are up-regulated in the aged brain and likely contribute significantly to brain aging. Finally, a subset of these genes whose expression is affected by aging are oppositely affected by exposure of mice to an enriched environment, suggesting that these genes may play important roles in learning and memory.

Although the molecular basis of aging remains unknown, a large body of evidence indicates that oxidative stress results in DNA damage that subsequently leads to changes in gene expression and organismal aging (1). Genetic modifications or spontaneous mutations in a variety of organisms that result in oxidative stress resistance increase longevity (2–4). In *Caenorhabditis elegans*, the reduction of metabolic rate through genetic manipulation or environmental changes increases life span (5). Dietary restriction also delays the aging process and extends life span (1), possibly by lowering metabolic rate and thus reducing the production of reactive oxygen species.

The hypothalamus plays a key role in regulating metabolism. Consequently, an understanding of the effects of aging on the hypothalamus may provide important insights into the organismal aging process. For example, it has been shown that neuroendocrine regulation of insulin signaling affects longevity in *C. elegans* (6–8). In mammals, hypothalamic modulation of the neuroendocrine system may regulate the aging process by controlling the production, processing, and degradation of neuroendocrine hormones and neuropeptides.

To better understand the molecular processes involved in aging, we have examined changes in gene expression in the aged hypothalamus. To determine whether these age-associated gene expression changes are tissue specific, we also analyzed gene expression in the cortex of young and aged mice. Our results demonstrate that the expression levels of many genes related to neuronal signaling, plasticity, and structure were changed in the aged brain. Moreover, many proteases were up-regulated during the aging process in both hypothalamus and cortex, a number of which are involved in the processing and degradation of neuropeptides. Altered expression of these genes may contribute to age-related disorders of synaptic plasticity and memory storage, neurodegenerative diseases, and normal organismal aging.

Materials and Methods

Tissues and RNA Preparation. Cortex and hypothalamus from three young (2 months) and three old (22 months) BALB/c mice were

dissected and immediately frozen in dry ice. The tissues were pooled and stored at -80°C . Total RNA was isolated from tissues by using the RNA Extraction Kit (Pharmacia-Biotech). Briefly, 60–120 mg of tissue was homogenized in 2 ml of prewarmed extraction buffer. The homogenate was centrifuged for 5 min at room temperature to remove cellular debris. The supernatant was transferred to a fresh sterile tube and then sheared by passing through a 23-gauge needle several times. This homogenate was layered on a cushion of cesium trifluoroacetate, then centrifuged overnight at $125,000 \times g$ at 15°C . After centrifugation, the supernatant was aspirated, and the RNA pellet at the bottom of the tube was resuspended, followed by ethanol precipitation. Samples were stored at -80°C .

High-Density Oligonucleotide Microarray Analysis. Double-stranded DNA was synthesized from 5 μg of total RNA by using the SuperScript Choice System (Life Technologies, Grand Island, NY) and a primer containing poly(dT) and a T7 RNA polymerase promoter sequence (Genset, La Jolla, CA). *In vitro* transcription by using double-stranded cDNA as a template in the presence of biotinylated UTP and CTP was carried out by using Enzo BioArray High Yield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA, and Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to the arrays in 100 mM Mes, pH 7.4/1 M NaCl/20 mM EDTA/0.01% Tween 20. The arrays were washed and stained with streptavidin-phycoerythrin and then scanned with an Affymetrix GeneArray Scanner. Data were analyzed with the Affymetrix GENECHIP EXPRESSION ANALYSIS software (version 3.1), as described (9). Labeled RNA samples were hybridized twice to two different arrays, and differences observed consistently in the replicates were analyzed further.

Results and Discussion

We have examined gene expression changes in the hypothalamus and cortex during the aging process by using high-density oligonucleotide arrays with probes for more than 11,000 genes (13,069 probe sets). The hypothalamus from young and aged mice showed altered expression of 99 genes [Table 1 and supplementary data (see www.pnas.org)], whereas the expression levels of 98 genes were changed in the cortex of aged mice (Table 2 and supplementary data). Twenty changes ($\approx 20\%$) were common to hypothalamus and cortex.

Age-Associated Expression of Metabolic Enzymes. The hypothalamus from aged mice showed increased expression levels of enzymes involved in mitochondria respiratory chain relative to young mice. The expression of four NADH-ubiquinone oxidoreductase subunits, two cytochrome *c* oxidase subunits, and three ATP synthase subunits was increased more than 2-fold. Mitochondrial respiration

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Table 1. Gene expression changes in the hypothalamus from young (2 months) and old (22 months) BALB/c mice

Accession number	Gene name	Fold change
Metabolic enzymes		
W33716	NADH-ubiquinone oxidoreductase KFYI subunit	2.8
Y07708	NADH oxidoreductase subunit MWFE	3
AA109866	NADH-ubiquinone oxidoreductase chain 4L	2.1
AA219829	NADH-ubiquinone oxidoreductase SGDh subunit	2.5
AA521794	Cytochrome c oxidase subunit VIIIb	2.2
AA672840	ATP synthase O subunit	2.2
C76507	ATP synthase γ -subunit	2.7
AA003458	Sarco/endoplasmic reticulum Ca²⁺-ATPase 2	-2.9
X56007	Na/K-ATPase β 2	-11
AA106307	H⁺ ATPase subunit E	-6.1
AA087605	H(+)-ATPase (mvp)	-6.9
Protein processing		
U05333	Cochaperonin 'cofactor A'	6.8
U09659	Mitochondrial chaperonin 10	2.6
Z31557	Chaperonin containing TCP-1	3.2
AA027544	Ubiquitin-activating enzyme E1	2.8
AA146437	Cathepsin S precursor, a cysteine proteinase	3.4
Z31297	Sorting nexin 2-like protein, protein degradation	4.8
AA013993	Prolyl olig-peptidase	11
AA020512	Caspase 6	3
Neuronal growth/structure		
U95116	Lissencephaly-1 protein (LIS-1)	3
V00835	Metallothionein-I	3.3
X61452	Cell division control-related protein 2b	2.8
AA709861	A-X actin-like protein	3.5
L31397	Dynamin	-3.5
U27106	Clathrin-associated AP-2	-5.7
U86090	Kinesin heavy chain	-2.4
Neuronal signaling		
AA271109	Protein phosphatase 1, regulatory subunit	3.8
X15373	Inositol-1,4,5-triphosphate receptor	3.5
X51468	Preprosomatostatin gene	3
D37792	Synaptotagmin 1	-17
D50621	PSD-95/SAP90A	-16
AA048604	Apolipoprotein E	-6.7
AA068956	Protein phosphatase PP2A	-6.1
W12204	Ca ²⁺ /calmodulin-dependent protein kinase II	-3.6
X61434	cAMP-dependent protein kinase C β	-3.1
X57497	Glutamate receptor 1	-2.7
AA710375	N-ethylmaleimide-sensitive factor-attachment receptor	-3.4
AB006361	Prostaglandin D synthetase	-5.6
Stress response		
M60798	Cu(2+)-Zn2+ superoxide dismutase	2.9
AA107471	DnaJ homolog 2	-6.4
AA166139	DNA repair protein	-10.4
AA033408	Damage-specific DNA-binding protein, DNA repair	-4.6
D89787	Hif like protein	-6.5
U27830	Stress-inducible protein, STI1	-4.9

Each RNA sample was hybridized twice to two different arrays, and fold change values are averages of the duplicate measurements. Positive values indicate an increase, and negative values indicate a decrease in gene expression. Genes in bold are differentially expressed in both aged cortex and hypothalamus.

generates reactive oxygen species (ROS) that are responsible for the damage of macromolecules and lead to aging (10). High levels of the expression of mitochondrial respiratory enzymes suggest that the production of hypothalamic ROS is increased in aged mice. The induced ROS production may further affect the aging process by altering hypothalamic regulation of physiological processes through the neuroendocrine system. No similar changes were found in aged cortex, suggesting that different brain tissues may age at different rates because of the tissue-specific metabolic rate variability or physiological environment differences.

In both the aged hypothalamus and cortex, the expression levels of many ATPases, including Na/K⁺ ATPase, Ca²⁺ ATPase, and H⁺ ATPase, were reduced 2- to 10-fold. Previously, it has been shown that the aging process affects calcium-dependent signaling processes in the brain (11). Reactive oxygen species have also been shown to affect the sarco/endoplasmic reticulum Ca²⁺ ATPase-dependent transport of calcium that regulates neuronal calcium signal pathways (12). Similarly, changes in expression of the Na/K⁺ ATPase can modify intracellular concentrations of calcium, regulate action potentials, and control neuronal activity in the brain

Table 2. Gene expression changes in the cortex from young (2 months) and old (22 months) BALB/c mice

Accession number	Gene name	Fold change
Metabolic enzymes		
M21285	Stearoyl-CoA desaturase	2.4
U27315	Adenine nucleotide translocase-1	2.2
M84145	Fumarylacetoacetate hydrolase	3.1
U13841	ATPase subunit E	-4.4
AA105755	Na ⁺ ,K ⁺ -ATPase α	-4.6
AA003458	Sarco/endoplasmic reticulum Ca²⁺-ATPase 2	-5.9
X56007	Na/K-ATPase β 2 subunit	-2.3
AA106307	H(+)-ATPase E-like protein	-3.8
AA389346	Citrate synthase	-3.4
Protein processing		
M13500	Kallikrein gene	5.2
X61232	Carboxypeptidase H	3.6
X92665	Ubiquitin-conjugating enzyme UbcM3	3
AA013993	Prolyl oligopeptidase	2.7
AA020512	Caspase 6	2.2
Z30970	Metalloproteinase-3 tissue inhibitor	2.1
Neuronal growth/structure		
U95116	Lissencephaly-1 protein	2.6
L20899	Cell division cycle (CDC25)	2.6
C76314	Cdc5-like protein	2.4
AA590859	A-X actin-like protein	7.3
U27106	Clathrin-associated AP-2	-5.5
AA118546	Actin-like protein 3	-6.2
AA050703	Defender against death 1 (DAD1)	-2.4
W18503	Dynein heavy chain, retrograde transport	-7.7
AA111631	Dynactin 1, retrograde axonal transport	-3.4
L31397	Dynamin	-2.7
Neuronal signaling		
AA168959	25 kDa FK506-binding protein FKBP25	2.7
L32372	AMPA receptor subunit (GluR-B)	2.8
X79082	MDK1, a receptor tyrosine kinase	2
D37792	Synaptotagmin I	-13.2
M73490	Apolipoprotein E	-6.9
AA124955	Casein kinase 1 α	-2.9
Z67745	Phosphatase 2A catalytic subunit	-6.2
M27073	Protein phosphatase 1 β	-2.7
J02626	cAMP-dependent protein kinase C β	-4.6
W13835	<i>N</i> -ethylmaleimide-sensitive membrane protein homolog	-3.2
U10120	<i>N</i> -ethylmaleimide sensitive factor	-10.3
AB006361	Prostaglandin D synthetase	-3.2
M27844	Calmodulin	-2.7
M63436	GABA-A receptor α -1 subunit	-7.1
Stress response		
AA105022	Heat-shock protein hsp84-like protein	-2.7
AA204094	HSP40/DNAJ homolog	-2.9

Each RNA sample was hybridized twice to two different arrays, and fold change values are averages of the duplicate measurements. Genes in bold are differentially expressed in both aged cortex and hypothalamus.

(13). Recently, it has been shown that H⁺ ATPase is also involved in the regulation of calcium signaling (14). Inhibition of H⁺ ATPase activity increases the intracellular calcium concentration and induces apoptosis (15). Thus, age-associated down-regulation of ATPases leads to changes in normal ion homeostasis, and that may affect neuronal signaling processes.

Alteration of Synaptic Protein Expression in the Aged Brain. Aging is associated with the down-regulation of many genes involved in synaptic transmission in both the hypothalamus and cortex. The expression levels of synaptotagmin I, a synaptic vesicle-associated protein that is involved in the regulation of neurotransmitter release, decreased 17-fold in the hypothalamus and 13-fold in the

cortex of aged mice. *N*-ethylmaleimide-sensitive factor-attachment receptor, a protein involved in synaptic vesicle exocytosis, was down-regulated 3.4-fold in hypothalamus and 10.3-fold in cortex of aged mice. Because these proteins play essential roles in regulating neurotransmitter release (16), down-regulation of these proteins could be responsible for the reduction of synaptic transmission at the nerve terminals in aged brain. Expression of cAMP-dependent protein kinase C β also decreased more than 3-fold in both the aged hypothalamus and cortex. This protein kinase plays an important role in synaptic plasticity and memory formation (17, 18), suggesting that down-regulation of the kinase may directly contribute to age-related memory deficits.

Although the expression levels of many synaptic proteins were

altered in both cortex and hypothalamus, some genes in this group were differentially regulated only in one brain region during the aging process (Tables 1 and 2). For example, expression of postsynaptic density protein-95, which plays a role in *N*-methyl-D-aspartate-dependent signaling, decreased 16-fold in the aged hypothalamus but not in the cortex. Expression of the GABA receptor α -1 subunit, which plays an essential role in the signal transduction of neurotransmitter GABA, decreased 7-fold in the aged cortex but not in the hypothalamus. These results suggest that there may be general as well as specific molecular pathways leading to synaptic modification in different brain regions during the aging process.

Altered Expression of Proteins Associated with Neuronal Structure in the Aged Brain. The expression of several genes associated with neuronal structure also changed in both the hypothalamus and cortex during aging. Dynamin and clathrin-associated AP-2 were down-regulated in both the aged hypothalamus and cortex. These two proteins play important roles in synaptic vesicle recycling and neuronal growth (19). The expression of lissencephaly-1 increases in the aged hypothalamus and cortex. Lissencephaly-1 is a phosphoprotein involved in neuronal migration and cortex development (20). Mutation of the gene in human results in a severe brain developmental disorder caused by abnormal neuronal migration; alteration of expression of lissencephaly-1 gene is also associated with brain developmental disorders (21). A-X actin is up-regulated in the brain of aged mice (Tables 1 and 2) and in Alzheimer's animal models (unpublished data). Although it may participate in the structural change in the brain, the physiological significance of A-X actin induction during the aging process is not clear.

Induction of Proteases in the Aged Brain. The expression of a number of proteins involved in protein processing increased in both the hypothalamus and cortex during the aging process, suggesting that protein processing and degradation may play an important role in brain aging. Some of the proteases are involved in the degradation of neuropeptides and hormones that are essential for normal brain function. The highest increase in expression is with prolyl oligopeptidase (11-fold in hypothalamus, 2.7-fold in cortex). This protease is responsible for the degradation of many neuropeptides and hormones, including gonadotropin-releasing hormone, substance P, arginine-vasopressin, and thyrotropin-releasing hormone (22). Degradation of hormones and other neuropeptides by prolyl oligopeptidase may accelerate the aging process and lead to age-related diseases; conversely, inhibition of the peptidase may have a beneficial effect on the aged brain. Indeed, inhibitors of prolyl oligopeptidase have been shown to prevent the deposition of β -amyloid in the hippocampus of the senescence-accelerated mouse (23). Moreover, a potent inhibitor of the peptidase, JTP-4819, improves learning and memory tasks in rodents and is being explored for the treatment of Alzheimer's disease (24).

Another protease that increases in the aged brain is caspase-6 (3-fold in hypothalamus, 2.2-fold in cortex). Caspase-6 belongs to a family of cysteine proteases that is involved in neuronal apoptosis (25). In addition, caspase-6 participates in the processing of amyloid precursor protein and the deposition of amyloid in the brain, which contributes significantly to age-related neurodegenerative diseases (26). Because neuronal apoptosis is closely associated with brain aging, other caspases that are involved in apoptosis may also be affected. Indeed, expression of caspase-2 and -3 were increased in the brain of Alzheimer's patients (27). Furthermore, the expression and activity of caspase-8 and -3 are altered in lymphocytes of aged humans (28).

Some proteases are up-regulated in only the aged hypothalamus or cortex. These include kallikrein (5.2-fold change in the cortex) and cathepsin S (3.4-fold change in the hypothalamus),

both of which are involved in degradation of neuropeptides and regulation of β -amyloid signaling (29). Cathepsin S is also up-regulated in the brain of Alzheimer's patients (30). Previously, cathepsin D, S, and Z were shown to be up-regulated in neocortex and cerebellum of old mice (31). Taken together, these results suggest that proteases involved in the degradation of neuropeptides and hormones, as well as caspases that are essential for neuronal apoptosis, may play important roles in brain aging. Studies by using animal models of overexpression or null mutations of these genes will help us to understand the functions of the proteases in brain aging. Furthermore, inhibitors of these proteases may provide effective therapeutic drugs for age-associated neurodegenerative diseases.

Expression of Proteins Associated with Age-Related Neurodegenerative Disorders. The expression level of apolipoprotein E decreased more than 5-fold in both the aged hypothalamus and cortex. In the brain, apolipoprotein E participates in the maintenance of synaptic integrity (32); expression of different isoforms of apolipoprotein E in the brain is known to affect cognitive performance and contribute to the development of Alzheimer's disease (33). Protein phosphatase 2A, a multifunctional enzyme involved in many physiological pathways, was also down-regulated 6-fold in the hypothalamus and cortex during aging. In the central nervous system, protein phosphatase 2A dephosphorylates τ protein (34); suppression of the activity of protein phosphatase 2A causes hyperphosphorylation of τ and participates in the pathogenesis of Alzheimer's disease (34). Thus down-regulation of protein phosphatase 2A may contribute to the formation of tangles in the aging brain. Finally, the down-regulation of prostaglandin D synthetase in the aged cortex and hypothalamus decreases the production of prostaglandin D2 in the brain. Because prostaglandin D2 is a potent endogenous hormone for promoting sleep (35), down-regulation of the prostaglandin D synthetase in the aged hypothalamus and cortex may contribute to the sleep disorders associated with aging.

Expression of Stress-Response Genes in the Aged Brain. Previously, it was reported that aging is associated with induction of stress-response genes in neocortex, cerebellum, and skeletal muscle in the mouse (31, 36). In contrast, we have found that, in the hypothalamus and cortex, a number of stress-response genes are down-regulated during the aging process. For example, DnaJ homolog 2, DNA repair protein, damage-specific DNA-binding protein, Hif-like protein, and stress-inducible protein STI1 were down-regulated from 4- to 10-fold in the aged hypothalamus. The decreased levels of DNA repair proteins in the aged brain correlate well with the fact that DNA repair efficiency is significantly lower in aged tissues (37, 38). In cortex, expression levels of heat-shock protein 84, like protein and heat-shock protein 40, decreased 3-fold during aging. It is also interesting to note that many inflammatory response genes were altered in the brain of 30-month-old mice (31), whereas we did not find this phenomenon in our 22-month-old mice. One explanation for the contradictory results is that the stress and inflammatory responses occur only at the latest stage of life in mice.

Opposite Effects on Gene Expression in Aged Versus Enriched Mice. In our previous work, we have identified genes whose expression was affected by enriched environmental training from cortex of CBA/B6 hybrid mice. In this study, we have shown that expression of some of these genes is also altered in the aged brain of BALB/c mice but in an opposite manner. It is known that aging is characterized by a decline in learning and memory capacity, whereas exposure of animals to an enriched environment can overcome the memory deficits of aged animals (39). It is possible that genes whose expression levels are oppositely regulated during enrichment training and aging may play important roles in learning and memory. For example, brain aging was associated with the down-regulation of

calmodulin (−2.7-fold). In contrast, calmodulin mRNA levels increased after 3 h (1.8-fold), 6 h (1.7-fold), 2 days (2.5-fold), and 14 days (2.4-fold) of training (40). Calmodulin, a calcium signaling protein, plays an essential role in the regulation of neuronal activity. It modulates the interaction of the postsynaptic density protein-95 protein complex with NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase (41). Calmodulin also regulates clustering of neurotransmitter receptors at central synapses (41). Similarly, gene expression of AP2 and synaptotagmin I decreased in the aged brain but increased after training. These proteins also play essential roles in synaptic transmission and plasticity.

Another protein, defender against cell death 1 (DAD1), was down-regulated (−2.4-fold) during aging but up-regulated (2.1-fold) after enriched environmental training (40). Mice harboring a disrupted *dad1* gene exhibit abnormal cell death, and the homozygous deleted mice die soon after implantation (42), implicating DAD1 in the control of programmed cell death during development. Our data suggest that down-regulation of DAD1 during aging might be involved in the increased neuronal death observed during the aging process (43); conversely, up-regulation of DAD1 after the enrichment training may play a neuroprotective role in the brain.

The expression of prostaglandin D synthetase was increased during the early phase of enrichment (2.1- and 2.6-fold at 3 and 6 h) (40) but decreased in the aged brain (−6-fold). As noted earlier, the down-regulation of prostaglandin D synthetase with age may contribute to age-associated sleep disorders (44). Although prostaglandin D synthetase also functions as a retinoic acid transporter, the physiological role of the induction as a result of environmental enrichment is not clear.

Our data also revealed that both aging and exposure to an

enriched environment are associated with the differential expression of specific proteases. For example, the expression of both prolyl oligopeptidase and caspase-6 is up-regulated in the aged brain but down-regulated after enrichment training. This observation reinforces the notion that protease misregulation may be a key contributor to age-related impairment of learning and memory.

Finally, a number of genes involved in neuronal growth and structure were oppositely regulated during aging and enrichment training. For example, the expression of Lissencephaly-1 was induced in aged brain but decreased in trained mice (−2.4 and −2.1-fold after 3 and 6 h, respectively). A cytoskeletal protein, dynactin, was down-regulated in the aged brain but up-regulated after enriched environmental training. In contrast, A-X actin expression increased in the aged brain but decreased after training. These proteins may be involved in the modulation of structural changes that occur during aging and enrichment (45).

Conclusions

We have shown that aging leads to the misregulation of many proteins involved in neuronal signaling and structure that may be associated with the age-related phenotype and diseases. In addition, alterations in protease levels likely play an important role in the aging brain as well as having a broader effect on the neuroendocrine system. Additional experiments to determine which of these changes are primary-cause factors in age-related brain dysfunction may provide new insights into the aging process and new approaches to the development of therapeutic agents.

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